



(86) Date de dépôt PCT/PCT Filing Date: 2001/02/09
(87) Date publication PCT/PCT Publication Date: 2001/08/16
(85) Entrée phase nationale/National Entry: 2002/07/24
(86) N° demande PCT/PCT Application No.: US 2001/004203
(87) N° publication PCT/PCT Publication No.: 2001/058494
(30) Priorités/Priorities: 2000/02/11 (60/181,743) US;
2000/06/23 (09/599,997) US; 2000/08/28 (60/228,337) US

(51) Cl.Int.⁷/Int.Cl.⁷ A61K 48/00, A61P 27/02, C07K 14/81,
C07K 14/515

(71) Demandeur/Applicant:
GEN-VEC, INC., US

(72) Inventeurs/Inventors:
KOVESDI, IMRE, US;
BROUGH, DOUGLAS E., US;
MCVEY, DUNCAN L., US;
WEI, LISA, US

(74) Agent: R. WILLIAM WRAY & ASSOCIATES

(54) Titre : MATIERES ET PROCEDES DESTINES AU TRAITEMENT DE TROUBLES OCULAIRES
(54) Title: MATERIALS AND METHODS FOR TREATING OCULA-RELATED DISORDERS

(57) **Abrégé/Abstract:**

The present invention is directed to a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, e.g., ocular neovascularization or age-related macular degeneration. The method comprises contacting an ocular cell with an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent. The method also can comprise contacting an ocular cell with different expression vectors, each comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a nucleic acid sequence encoding a neurotrophic agent. In addition, the present invention provides a viral vector comprising a nucleic acid sequence encoding pigment epithelium-derived factor (PEDF) or a therapeutic fragment thereof.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 August 2001 (16.08.2001)

PCT

(10) International Publication Number
WO 01/58494 A3

(51) International Patent Classification⁷: **A61K 48/00**,
C07K 14/81, 14/515, A61P 27/02

(74) Agents: **KILYK, John, Jr.** et al.; Leydig, Voit & Mayer,
Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson,
Chicago, IL 60601-6780 (US).

(21) International Application Number: PCT/US01/04203

(22) International Filing Date: 9 February 2001 (09.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/181,743 11 February 2000 (11.02.2000) US
09/599,997 23 June 2000 (23.06.2000) US
60/228,337 28 August 2000 (28.08.2000) US

(71) Applicant (*for all designated States except US*): **GEN-VEC, INC.** [US/US]; 65 West Watkins Mill Road, Gaithersburg, MD 20878 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **KOVESDI, Imre** [CA/US]; 7713 Warbler Lane, Rockville, MD 20855 (US). **BROUGH, Douglas, E.** [US/US]; 3900 Shallowbrook Lane, Olney, MD 20832 (US). **MCVEY, Duncan, L.** [US/US]; 6016 Muncaster Mill Road, Derwood, MD 20855 (US). **WEI, Lisa** [US/US]; 14109 Saddle River Drive, Gaithersburg, MD 20878 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:
4 April 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: GENE THERAPY FOR TREATING OCULAR-RELATED DISORDERS

(57) **Abstract:** The present invention is directed to a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, e.g., ocular neovascularization or age-related macular degeneration. The method comprises contacting an ocular cell with an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent. The method also can comprise contacting an ocular cell with different expression vectors, each comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a nucleic acid sequence encoding a neurotrophic agent. In addition, the present invention provides a viral vector comprising a nucleic acid sequence encoding pigment epithelium-derived factor (PEDF) or a therapeutic fragment thereof.

WO 01/58494 A3



MATERIALS AND METHODS FOR TREATING OCULAR-RELATED DISORDERS

FIELD OF THE INVENTION

[0001] The present invention relates to a method of prophylactically or therapeutically treating an ocular disorder as well as materials useful for treating an ocular disorder.

BACKGROUND OF THE INVENTION

[0002] An overwhelming majority of the world's population will experience some degree of vision loss in their lifetime. Vision loss affects virtually all people regardless of age, race, economic or social status, or geographical location. Ocular-related disorders, while often not life threatening, necessitate life-style changes that jeopardize the independence of the afflicted individual. Vision impairment can result from most all ocular disorders, including diabetic retinopathies, proliferative retinopathies, retinal detachment, toxic retinopathies, retinal vascular diseases, retinal degenerations, vascular anomalies, age-related macular degeneration and other acquired disorders, infectious diseases, inflammatory diseases, ocular ischemia, pregnancy-related disorders, retinal tumors, choroidal tumors, choroidal disorders, vitreous disorders, trauma, cataract complications, dry eye, and inflammatory optic neuropathies.

[0003] Leading causes of severe vision loss and blindness are ocular-related disorders wherein the vasculature of the eye is damaged or insufficiently regulated. Ocular-related diseases comprising a neovascularization aspect are many and include, for example, exudative age-related macular degeneration, diabetic retinopathy, corneal neovascularization, choroidal neovascularization, neovascular glaucoma, cyclitis, Hippel-Lindau Disease, retinopathy of prematurity, pterygium, histoplasmosis, iris neovascularization, macular edema, glaucoma-associated neovascularization, and the like. It is likely that severe vision loss does not result directly from neovascularization, but the effect of neovascularization on the retina. The retina is a delicate ocular membrane on which images are received. Near the center of the retina is the macula lutea, an oval area of retinal tissue where visual sense is most acute. The retina is most delicate at the fovea centralis, the central depression located in the center of the macula. Damage of the retina, i.e., retinal detachment, retinal tears, or retinal degeneration, is directly connected to vision loss. While a common cause of retinal detachment, retinal tears, and retinal degeneration is abnormal, i.e., uncontrolled, vascularization of various ocular tissues, this is not always the case. Atrophic complications associated with age-related macular degeneration, nonproliferative diabetic retinopathy, and inflammatory ocular damage are not associated with neovascularization, but can result in severe vision loss if not treated. Disorders

associated with both neovascular and atrophic components, such as age-related macular degeneration and diabetic retinopathy, are particularly difficult to treat due to the emergence of a wide variety of complications.

[0004] Age-related macular degeneration is the leading cause of blindness in patients over 65 years of age. As the elderly population of the world increases, the incidence of age-related macular degeneration is expected to increase dramatically, reaching a predicted 7.5 million cases in the United States alone by the year 2030 (Hyman et al., *Am. J. Epidemiol.*, 118, 213-227 (1983)). Age-related macular degeneration (AMD) is a progressive, degenerative disorder of the eye resulting initially in loss of visual acuity. Complications arising with advanced age-related macular degeneration include atrophic and exudative complications. Atrophic complications stem from retinal pigment epithelial cell loss resulting in atrophy of the retinal pigment epithelium (RPE). Exudative complications include disciform scars (i.e., scarring involving fibrous elements) and neovascularization. Severe vision loss occurs as neovascularization or atrophy disturbs the foveal center (Bressler et al., *Ophthalmology*, 102, 1206-1211 (1995)). Ultimately, blindness from age-related macular degeneration stems from degeneration of the RPE and the subsequent death of photoreceptors.

[0005] Similarly, diabetic retinopathy is subdivided into a nonproliferative stage, which typically occurs first, and a proliferative stage. Vision loss associated with nonproliferative diabetic retinopathy stems from retinal edema, in particular diabetic macular edema, resulting from vascular leakage. Focal and diffuse vascular leakage occurs as a result of microvascular abnormalities, intraretinal microaneurysms, capillary closure, and retinal hemorrhages. Prolonged periods of vascular leakage ultimately lead to thickening of the basement membrane and formation of soft and hard exudates. Nonproliferative diabetic retinopathy is also characterized by loss of retinal pericytes. The proliferative stage of diabetic retinopathy is characterized by neovascularization and fibrovascular growth (i.e., scarring involving glial and fibrous elements) from the retina or optic nerve over the inner surface of the retina or disc or into the vitreous cavity. Retinal neovascularization is the leading cause of vision loss associated with proliferative diabetic retinopathy.

[0006] For many ocular-related disorders, no efficient therapeutic options currently are available. Laser photocoagulation involves administering laser burns to various areas of the eye and is used in the treatment of many neovascularization-linked disorders. For example, focal macular photocoagulation is used to treat areas of vascular leakage in the macula (Murphy, *Amer. Family Physician*, 51(4), 785-796 (1995)). Similarly, neovascularization, in particular, advanced proliferative retinopathy, is commonly treated with scatter or panretinal photocoagulation. However, laser treatment may cause permanent blind spots

corresponding to the treated areas. Laser treatment may also cause persistent or recurrent hemorrhage, increase the risk of retinal detachment, or induce neovascularization or fibrosis. With respect to age-related macular degeneration, many patients eventually experience severe vision loss in spite of treatment. Other treatment options for ocular-related disorders include thermotherapy, radiation therapy, surgery, e.g., macular translocation, removal of excess ocular tissue, and the like. However, in most cases, all available treatment options have limited therapeutic effect, require repeated, costly procedures, and/or are associated with dangerous side-effects.

[0007] Given the prevalence of ocular-related disorders, there remains a need for an effective prophylactic and therapeutic treatment of ocular-related disorders, in particular those ocular-related disorders associated with both atrophic and neovascular complications, such as diabetic retinopathy and age-related macular degeneration. Accordingly, the present invention provides materials and methods for prophylactically and therapeutically treating ocular-related disorders, including treatment of nonproliferative complications and proliferative complications. This and other advantages of the present invention will become apparent from the detailed description provided herein.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention provides a method for the prophylactic or therapeutic treatment of ocular-related disorders. In particular, the present invention provides a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, such as ocular neovascularization and age-related macular degeneration. The method comprises contacting an ocular cell with (a) an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent, or (b) different expression vectors, each comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a neurotrophic agent. The nucleic acid sequence encoding the inhibitor of angiogenesis and/or the nucleic acid sequence encoding the neurotrophic agent is (are) expressed, thereby resulting in the production of the inhibitor of angiogenesis and/or the neurotrophic agent to prophylactically or therapeutically treat the animal for an ocular-related disorder. Preferably, the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent are the same nucleic acid sequence. More preferably, the nucleic acid sequence encodes a factor comprising both anti-angiogenic and neurotrophic activity. Most preferably, the factor is PEDF.

[0009] In addition, the present invention provides a viral vector comprising a nucleic acid sequence encoding pigment epithelium-derived factor (PEDF) or a

therapeutic fragment thereof. The nucleic acid sequence is operably linked to regulatory sequences necessary for expression of PEDF or a therapeutic fragment thereof. Preferably, the viral vector is an adenoviral vector or an adeno-associated viral vector. Also preferably, the viral vector further comprises one or more additional nucleic acid sequences encoding therapeutic substances other than PEDF or a therapeutic fragment thereof.

DETAILED DESCRIPTION OF THE INVENTION

[0010] The present invention is directed to methods of prophylactically or therapeutically treating an animal, preferably a human, for at least one ocular-related disorder. The present invention also provides materials for treating ocular-related disorders. Ocular-related disorders appropriate for treatment using the present inventive materials and methods include, but are not limited to, diabetic retinopathies, proliferative retinopathies, retinopathy of prematurity, retinal vascular diseases, vascular anomalies, age-related macular degeneration and other acquired disorders, endophthalmitis, infectious diseases, inflammatory diseases, AIDS-related disorders, ocular ischemia syndrome, pregnancy-related disorders, peripheral retinal degenerations, retinal degenerations, toxic retinopathies, cataracts, retinal tumors, corneal neovascularization, choroidal tumors, choroidal disorders, choroidal neovascularization, neovascular glaucoma, vitreous disorders, retinal detachment and proliferative vitreoretinopathy, cyclitis, non-penetrating trauma, penetrating trauma, post-cataract complications, Hippel-Lindau Disease, dry eye, inflammatory optic neuropathies, macular edema, pterygium, iris neovascularization, surgical-induced disorders, and the like.

[0011] In particular, the present invention provides a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, such as ocular neovascularization. The method comprises contacting an ocular cell with an expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic agent. Preferably, the method comprises contacting an ocular cell with an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent. Desirably, the nucleic acid sequence encodes at least one inhibitor of angiogenesis and at least one neurotrophic agent. The ocular neovascularization treated by the present inventive method can be neovascularization of the choroid. The choroid is a thin, vascular membrane located under the retina. Abnormal neovascularization of the choroid results from, for example, photocoagulation, anterior ischemic optic neuropathy, Best's disease, choroidal hemangioma, metallic intraocular foreign body, choroidal

nonperfusion, choroidal osteomas, choroidal rupture, bacterial endocarditis, choroideremia, chronic retinal detachment, drusen, deposit of metabolic waste material, endogenous *Candida* endophthalmitis, neovascularization at ora serrata, operating microscope burn, punctate inner choroidopathy, radiation retinopathy, retinal cryoinjury, retinitis pigmentosa, retinochoroidal coloboma, rubella, subretinal fluid drainage, tilted disc syndrome, *Taxoplasma* retinochoroiditis, tuberculosis, and the like.

[0012] Neovascularization of the cornea is also appropriate for treatment by the method of the present invention. The cornea is a projecting, transparent section of the fibrous tunic, the outer most layer of the eye. The outermost layer of the cornea contacts the conjunctiva, while the innermost layer comprises the endothelium of the anterior chamber. Corneal neovascularization stems from, for example, ocular injury, surgery, infection, improper wearing of contact lenses, and diseases such as, for example, corneal dystrophies.

[0013] Alternatively, the ocular neovascularization is preferably neovascularization of the retina. Retinal neovascularization is an indication associated with numerous ocular diseases and disorders, many of which are named above. Preferably, the neovascularization of the retina treated in accordance with the present inventive method is associated with diabetic retinopathy. Common causes of retinal neovascularization include ischemia, viral infection, and retinal damage. Neovascularization of the retina can lead to macular edema, subretinal discoloration, scarring, and the like. Complications associated with retina neovascularization stem from breakage and leakage of newly formed blood vessels. Vision is impaired as blood fills the vitreous cavity and is not efficiently removed. Not only is the passage of light impeded, but an inflammatory response to the excess blood and metabolites can cause further damage to ocular tissue. In addition, the new vessels form fibrous scar tissue, which, over time, will disturb the retina causing retinal tears and detachment.

[0014] The present invention also provides a method for prophylactically or therapeutically treating an animal for age-related macular degeneration. The method comprises contacting an ocular cell associated with age-related macular degeneration with an expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Desirably, the expression vector comprises a nucleic acid sequence encoding an inhibitor of angiogenesis and a nucleic acid sequence encoding a neurotrophic agent. More desirably, the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent are the same nucleic acid sequence. Preferably, the age-related macular degeneration is associated with at least one exudative complication. Exudative complications include, for example, disciform scars (i.e., scarring involving fibrous elements) and neovascularization. Alternatively, the age-related macular degeneration is

associated with at least one atrophic complication. Atrophic complications include, for instance, the formation of drusen and basal laminar deposits, irregularity of retinal pigmentation, and accumulation of lipofuscin granules.

[0015] By “prophylactic” is meant the protection, in whole or in part, against ocular-related disorders, in particular ocular neovascularization or age-related macular degeneration. By “therapeutic” is meant the amelioration of the ocular-related disorder, itself, and the protection, in whole or in part, against further ocular-related disease, in particular ocular neovascularization or age-related macular degeneration. One of ordinary skill in the art will appreciate that any degree of protection from, or amelioration of, an ocular-related disorder is beneficial to a patient. The present invention is particularly advantageous in that a therapeutic agent can be directly applied to affected areas without the harmful side effects of presently employed therapies.

[0016] The present inventive methods are useful in the treatment of both acute and persistent, progressive ocular-related disorders. For acute ailments, the expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor can be administered using a single or multiple applications within a short time period. For persistent ocular-related diseases, such as age-related macular degeneration and diabetic retinopathy, numerous applications of the expression vector may be necessary to realize a therapeutic effect.

[0017] One of ordinary skill in the art will appreciate that any of a number of expression vectors known in the art are suitable for use in the present inventive methods. Examples of suitable expression vectors include, for instance, plasmids, plasmid-liposome complexes, and viral vectors, e.g., parvoviral-based vectors (i.e., adeno-associated virus (AAV)-based vectors), retroviral vectors, herpes simplex virus (HSV)-based vectors, AAV-adenoviral chimeric vectors, and adenovirus-based vectors. Any of these expression vectors can be prepared using standard recombinant DNA techniques described in, e.g., Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

[0018] Plasmids, genetically engineered circular double-stranded DNA molecules, can be designed to contain an expression cassette for delivery of the nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor to an ocular cell. Although plasmids were the first vector described for administration of therapeutic nucleic acids, the level of transfection efficiency is poor compared with other techniques. By complexing the plasmid with liposomes, the efficiency of gene transfer in general is improved. While the liposomes used for plasmid-mediated gene transfer

strategies have various compositions, they are typically synthetic cationic lipids.

Advantages of plasmid-liposome complexes include their ability to transfer large pieces of DNA encoding a therapeutic nucleic acid and their relatively low immunogenicity.

[0019] Plasmids are often used for short-term expression. However, a plasmid construct can be modified to obtain prolonged expression. It has recently been discovered that the inverted terminal repeats (ITR) of parvovirus, in particular adeno-associated virus (AAV), are responsible for the high-level persistent nucleic acid expression often associated with AAV (see, for example, U.S. Patent 6,165,754). Accordingly, the expression vector can be a plasmid comprising native parvovirus ITRs to obtain prolonged and substantial expression of at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. While plasmids are suitable for use in the present inventive methods, preferably the expression vector is a viral vector.

[0020] AAV vectors are viral vectors of particular interest for use in gene therapy protocols. AAV is a DNA virus, which is not known to cause human disease. AAV requires co-infection with a helper virus (i.e., an adenovirus or a herpes virus), or expression of helper genes, for efficient replication. AAV vectors used for administration of a therapeutic nucleic acid have approximately 96% of the parental genome deleted, such that only the terminal repeats (ITRs), which contain recognition signals for DNA replication and packaging, remain. This eliminates immunologic or toxic side effects due to expression of viral genes. In addition, delivering the AAV *rep* protein enables integration of the AAV vector comprising AAV ITRs into a specific region of genome, if desired. Host cells comprising an integrated AAV genome show no change in cell growth or morphology (see, for example, U.S. Patent 4,797,368). Although efficient, the need for helper virus or helper genes can be an obstacle for widespread use of this vector.

[0021] Retrovirus is an RNA virus capable of infecting a wide variety of host cells. Upon infection, the retroviral genome integrates into the genome of its host cell and is replicated along with host cell DNA, thereby constantly producing viral RNA and any nucleic acid sequence incorporated into the retroviral genome. When employing pathogenic retroviruses, e.g., human immunodeficiency virus (HIV) or human T-cell lymphotropic viruses (HTLV), care must be taken in altering the viral genomic to eliminate toxicity. A retroviral vector can additionally be manipulated to render the virus replication-incompetent. As such, retroviral vectors are thought to be particularly useful for stable gene transfer *in vivo*. Lentiviral vectors, such as HIV-based vectors, are exemplary of retroviral vectors used for gene delivery. Unlike other retroviruses, HIV-based vectors are known to incorporate their passenger genes into non-dividing cells and, therefore, can be of use in treating atrophic forms of ocular-related disease.

[0022] HSV-based viral vectors are suitable for use as an expression vector to introduce nucleic acids into ocular cells. The mature HSV virion consists of an enveloped icosahedral capsid with a viral genome consisting of a linear double-stranded DNA molecule that is 152 kb. Most replication-deficient HSV vectors contain a deletion to remove one or more intermediate-early genes to prevent replication. Advantages of the herpes vector are its ability to enter a latent stage that can result in long-term DNA expression, and its large viral DNA genome that can accommodate exogenous DNA up to 25 kb. Of course, this ability is also a disadvantage in terms of short-term treatment regimens. For a description of HSV-based vectors appropriate for use in the present inventive methods, see, for example, U.S. Patents 5,837,532; 5,846,782; 5,849,572; and 5,804,413 and International Patent Applications WO 91/02788, WO 96/04394, WO 98/15637, and WO 99/06583.

[0023] Adenovirus (Ad) is a 36 kb double-stranded DNA virus that efficiently transfers DNA *in vivo* to a variety of different target cell types. For use in the present inventive methods, the virus is preferably made replication deficient by deleting select genes required for viral replication. The expendable E3 region is also frequently deleted to allow additional room for a larger DNA insert. The vector can be produced in high titers and can efficiently transfer DNA to replicating and non-replicating cells. The newly transferred genetic information remains epi-chromosomal, thus eliminating the risks of random insertional mutagenesis and permanent alteration of the genotype of the target cell. However, if desired, the integrative properties of AAV can be conferred to adenovirus by constructing an AAV-Ad chimeric vector. For example, the AAV ITRs and nucleic acid encoding the Rep protein incorporated into an adenoviral vector enables the adenoviral vector to integrate into a mammalian cell genome. Therefore, AAV-Ad chimeric vectors are an interesting option for use in the present invention.

[0024] Preferably, the expression vector of the present inventive methods is a viral vector; more preferably, the expression vector is an adenoviral vector. In the context of the present invention, the adenoviral vector can be derived from any serotype of adenovirus. Adenoviral stocks that can be employed as a source of adenovirus can be amplified from the adenoviral serotypes 1 through 51, which are currently available from the American Type Culture Collection (ATCC, Manassas, VA), or from any other serotype of adenovirus available from any other source. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, and 31), subgroup B (e.g., serotypes 3, 7, 11, 14, 16, 21, 34, and 35), subgroup C (e.g., serotypes 1, 2, 5, and 6), subgroup D (e.g., serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, and 42-47), subgroup E (serotype 4), subgroup F (serotypes 40 and 41), or any other adenoviral serotype. Preferably, however, an adenovirus is of

serotype 2, 5 or 9. However, non-group C adenoviruses can be used to prepare replication-deficient adenoviral gene transfer vectors for delivery of anti-angiogenic factors and/or neurotrophic factors to ocular cells. Preferred adenoviruses used in the construction of non-group C adenoviral gene transfer vectors include Ad12 (group A), Ad7 (group B), Ad30 and Ad36 (group D), Ad4 (group E), and Ad41 (group F). Non-group C adenoviral vectors, methods of producing non-group C adenoviral vectors, and methods of using non-group C adenoviral vectors are disclosed in, for example, U.S. Patents 5,801,030; 5,837,511; and 5,849,561 and International Patent Applications WO 97/12986 and WO 98/53087.

[0025] The adenoviral vector is preferably deficient in at least one gene function required for viral replication, thereby resulting in a “replication-deficient” adenoviral vector. Preferably, the adenoviral vector is deficient in at least one essential gene function of the E1 region of the adenoviral genome required for viral replication. In addition to a deficiency in the E1 region, the recombinant adenovirus can also have a mutation in the major late promoter (MLP). The mutation in the MLP can be in any of the MLP control elements such that it alters the responsiveness of the promoter, as discussed in International Patent Application WO 00/00628. More preferably, the vector is deficient in at least one essential gene function of the E1 region and at least part of the E3 region (e.g., an Xba I deletion of the E3 region). With respect to the E1 region, the adenoviral vector can be deficient in at least part of the E1a region and at least part of the E1b region. Preferably, the adenoviral vector is “multiply deficient,” meaning that the adenoviral vector is deficient in one or more essential gene functions required for viral replication in each of two or more regions. For example, the aforementioned E1-deficient or E1-, E3-deficient adenoviral vectors can be further deficient in at least one essential gene of the E4 region. Adenoviral vectors deleted of the entire E4 region can elicit lower host immune responses.

[0026] Alternatively, the adenoviral vector lacks all or part of the E1 region and all or part of the E2 region. However, adenoviral vectors lacking all or part of the E1 region, all or part of the E2 region, and all or part of the E3 region also are contemplated herein. In one embodiment, the adenoviral vector lacks all or part of the E1 region, all or part of the E2 region, all or part of the E3 region, and all or part of the E4 region. Suitable replication-deficient adenoviral vectors are disclosed in U.S. Patents 5,851,806 and 5,994,106 and International Patent Applications WO 95/34671 and WO 97/21826. For example, suitable replication-deficient adenoviral vectors include those with at least a partial deletion of the E1a region, at least a partial deletion of the E1b region, at least a partial deletion of the E2a region, and at least a partial deletion of the E3 region. Alternatively, the replication-deficient adenoviral vector can have at least a partial deletion of the E1 region, at least a partial deletion of the E3 region, and at least a partial deletion of the E4 region. Such

multiply-deficient viral vectors are particularly useful in that such vectors can accept large inserts of exogenous DNA. Indeed, adenoviral amplicons, an example of a multiply-deficient adenoviral vector which comprises only those genomic sequences required for packaging and replication of the viral genome, can accept inserts of approximately 36 kb.

[0027] Therefore, in a preferred embodiment, the expression vector of the present inventive method is a multiply-deficient adenoviral vector lacking all or part of the E1 region, all or part of the E3 region, all or part of the E4 region, and, optionally, all or part of the E2 region. In this regard, it has been observed that an at least E4-deficient adenoviral vector expresses a transgene at high levels for a limited amount of time *in vivo* and that persistence of expression of a transgene in an at least E4-deficient adenoviral vector can be modulated through the action of a trans-acting factor, such as HSV ICP0, Ad pTP, CMV-IE2, CMV-IE86, HIV tat, HTLV-tax, HBV-X, AAV Rep 78, the cellular factor from the U205 osteosarcoma cell line that functions like HSV ICP0, or the cellular factor in PC12 cells that is induced by nerve growth factor, among others. In view of the above, the multiply deficient adenoviral vector (e.g., the at least E4-deficient adenoviral vector) preferably further comprises a nucleic acid sequence encoding a trans-acting factor that modulates the persistence of expression of the nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Alternatively, the ocular cell is contacted with a second expression vector comprising a nucleic acid sequence encoding a trans-acting factor that modulates the persistence of expression of the nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Preferably, the nucleic acid sequence encoding the trans-acting factor does not encode an adenoviral E4 region gene product. Whether expressed from the adenoviral vector or supplied by a second expression vector, preferably, the trans-acting factor is the *Herpes simplex* infected cell polypeptide 0 (HSV ICP0).

[0028] It should be appreciated that the deletion of different regions of the adenoviral vector can alter the immune response of the mammal. In particular, deletion of different regions can reduce the inflammatory response generated by the adenoviral vector. Furthermore, the adenoviral vector's coat protein can be modified so as to decrease the adenoviral vector's ability or inability to be recognized by a neutralizing antibody directed against the wild-type coat protein, as described in International Patent Application WO 98/40509. Such modifications are useful for long-term treatment of persistent ocular disorders.

[0029] Similarly, the coat protein of a viral vector, preferably an adenoviral vector, can be manipulated to alter the binding specificity or recognition of a virus for a viral receptor on a potential host cell. For adenovirus, such manipulations can include deletion of regions

of the fiber, penton, or hexon, insertions of various native or non-native ligands into portions of the coat protein, and the like. Manipulation of the coat protein can broaden the range of cells infected by a viral vector or enable targeting of a viral vector to a specific cell type. For example, in one embodiment, the expression vector is a viral vector comprising a chimeric coat protein (e.g., a fiber, hexon pIX, pIIIa, or penton protein), which differs from the wild-type (i.e., native) coat protein by the introduction of a nonnative amino acid sequence, preferably at or near the carboxyl terminus. Preferably, the nonnative amino acid sequence is inserted into or in place of an internal coat protein sequence. One of ordinary skill in the art will understand that the nonnative amino acid sequence can be inserted within the internal coat protein sequence or at the end of the internal coat protein sequence. The resultant chimeric viral coat protein is able to direct entry into cells of the viral, i.e., adenoviral, vector comprising the coat protein that is more efficient than entry into cells of a vector that is identical except for comprising a wild-type viral coat protein rather than the chimeric viral coat protein. Preferably, the chimeric virus coat protein binds a novel endogenous binding site present on the cell surface that is not recognized, or is poorly recognized by a vector comprising a wild-type coat protein. One direct result of this increased efficiency of entry is that the virus, preferably, the adenovirus, can bind to and enter numerous cell types which a virus comprising wild-type coat protein typically cannot enter or can enter with only a low efficiency.

[0030] In another embodiment of the present invention, the expression vector is a viral vector comprising a chimeric virus coat protein not selective for a specific type of eukaryotic cell. The chimeric coat protein differs from the wild-type coat protein by an insertion of a nonnative amino acid sequence into or in place of an internal coat protein sequence. In this embodiment, the chimeric virus coat protein efficiently binds to a broader range of eukaryotic cells than a wild-type virus coat, such as described in International Patent Application WO 97/20051.

[0031] Specificity of binding of an adenovirus to a given cell can also be adjusted by use of an adenovirus comprising a short-shafted adenoviral fiber gene, as discussed in U.S. Patent 5,962,311. Use of an adenovirus comprising a short-shafted adenoviral fiber gene reduces the level or efficiency of adenoviral fiber binding to its cell-surface receptor and increases adenoviral penton base binding to its cell-surface receptor, thereby increasing the specificity of binding of the adenovirus to a given cell. Alternatively, use of an adenovirus comprising a short-shafted fiber enables targeting of the adenovirus to a desired cell-surface receptor by the introduction of a nonnative amino acid sequence either into the penton base or the fiber knob.

[0032] Of course, the ability of a viral vector to recognize a potential host cell can be modulated without genetic manipulation of the coat protein. For instance, complexing an adenovirus with a bispecific molecule comprising a penton base-binding domain and a domain that selectively binds a particular cell surface binding site enables one of ordinary skill in the art to target the vector to a particular cell type.

[0033] Suitable modifications to a viral vector, specifically an adenoviral vector, are described in U.S. Patents 5,559,099; 5,731,190; 5,712,136; 5,770,442; 5,846,782; 5,926,311; 5,965,541; 6,057,155; 6,127,525; and 6,153,435 and International Patent Applications WO 96/07734, WO 96/26281, WO 97/20051, WO 98/07865, WO 98/07877, WO 98/54346, and WO 00/15823. Similarly, it will be appreciated that numerous expression vectors are available commercially. Construction of expression vectors is well understood in the art. Adenoviral vectors can be constructed and/or purified using the methods set forth, for example, in U.S. Patent 5,965,358 and International Patent Applications WO 98/56937, WO 99/15686, and WO 99/54441. Adeno-associated viral vectors can be constructed and/or purified using the methods set forth, for example, in U.S. Patent 4,797,368 and Laughlin et al., *Gene*, 23, 65-73 (1983).

[0034] The selection of expression vector for use in the present inventive method will depend on a variety of factors such as, for example, the host, immunogenicity of the vector, the desired duration of protein production, and the like. As each type of expression vector has distinct properties, a researcher has the freedom to tailor the present inventive method to any particular situation. Moreover, more than one type of expression vector can be used to deliver the nucleic acid sequence to the ocular cell. Thus, the present invention provides a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, wherein the method comprises contacting an ocular cell with different expression vectors, each comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a nucleic acid sequence encoding a neurotrophic agent. The nucleic acid sequence encoding the inhibitor of angiogenesis and/or the nucleic acid sequence encoding the neurotrophic agent are expressed, thereby resulting in the production of the inhibitor of angiogenesis and/or the neurotrophic agent to prophylactically or therapeutically treat the animal for an ocular-related disorder.

[0035] Preferably, at least two different types of expression vector (i.e., a plasmid and a viral vector or two different viral vectors) are delivered to the ocular cell. At least one expression vector can comprise a nucleic acid sequence encoding an inhibitor of angiogenesis. Similarly, at least one expression vector can comprise a nucleic acid sequence encoding a neurotrophic agent. Indeed, a mixture of expression vectors, some comprising the coding sequence for an inhibitor of angiogenesis and some comprising the

coding sequence for a neurotrophic agent, can be administered. Desirably, at least one expression vector comprises the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent. More preferably, the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent are the same nucleic acid. Also preferably, the inhibitor of angiogenesis and the neurotrophic agent are a single factor. Preferably, the ocular cell is contacted with an adenoviral vector and an adeno-associated viral vector. One of ordinary skill in the art will appreciate the ability to capitalize on the advantageous properties of multiple delivery systems to treat or study ocular-related disorders.

[0036] One embodiment of the present invention provides a method for prophylactically or therapeutically treating an animal for age-related macular degeneration. The method comprises contacting an ocular cell associated with age-related macular degeneration with an expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Alternatively, the ocular cell is contacted with at least two different types of expression vector, each expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. As age-related macular degeneration commonly afflicts the elderly, preferably the expression vector is administered to an animal, i.e., a human, at least 55 years old. Age-related macular degeneration is a complex disease associated with a wide variety of complications affecting a number of ocular tissues. Ocular cells associated with age-related macular degeneration include, but are not limited to, cells of neural origin, cells of all layers of the retina, especially retinal pigment epithelial cells, glial cells, and pericytes. Other ocular cells that are suitable for use in the method of the present invention include, for example, endothelial cells, iris epithelial cells, corneal cells, ciliary epithelial cells, Mueller cells, astrocytes, and cells of the trabecular meshwork. The trabecular meshwork is associated with the passage for fluid drainage out of the eye. Other cells linked to various ocular-related diseases include, for example, fibroblasts and vascular endothelial cells. In that a great deal of retinal damage occurs as a result of edema, thickening of underlying membranes, and build-up of metabolic byproducts, preferably the expression vector is administered to an area of vascular leakage.

[0037] According to the invention, the nucleic acid sequence is operably linked to regulatory sequences necessary for expression, i.e., a promoter. A “promoter” is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. A nucleic acid sequence is “operably linked” to a promoter when the promoter is capable of directing transcription of that nucleic acid sequence. A promoter can be native or non-native to the nucleic acid sequence to which it is operably linked.

[0038] Any promoter (i.e., whether isolated from nature or produced by recombinant DNA or synthetic techniques) can be used in connection with the present invention to provide for transcription of the nucleic acid sequence. The promoter preferably is capable of directing transcription in a eukaryotic (desirably mammalian) cell. The functioning of the promoter can be altered by the presence of one or more enhancers and/or silencers present on the vector. "Enhancers" are cis-acting elements of DNA that stimulate or inhibit transcription of adjacent genes. An enhancer that inhibits transcription also is termed a "silencer." Enhancers differ from DNA-binding sites for sequence-specific DNA binding proteins found only in the promoter (which also are termed "promoter elements") in that enhancers can function in either orientation, and over distances of up to several kilobase pairs (kb), even from a position downstream of a transcribed region.

[0039] A comparison of promoter sequences that function in eukaryotes has revealed conserved sequence elements. Generally, eukaryotic promoters transcribed by RNA polymerase II are typified by a "TATA box" centered at approximately position -25, which appears to be essential for accurately positioning the start of transcription. The TATA box directs RNA polymerase to begin transcribing approximately 30 base pairs (bp) downstream in mammalian systems. The TATA box functions in conjunction with at least two other upstream sequences located about 40 bp and 110 bp upstream of the start of transcription. Typically, a so-called "CCAAT box" serves as one of the two upstream sequences, and the other often is a GC-rich segment. The CCAAT homology can reside on different strands of the DNA. The upstream promoter element also can be a specialized signal such as one of those which have been described in the art and which appear to characterize a certain subset of genes.

[0040] To initiate transcription, the TATA box and the upstream sequences are each recognized by regulatory proteins which bind to these sites, and activate transcription by enabling RNA polymerase II to bind the DNA segment and properly initiate transcription. Whereas base changes outside the TATA box and the upstream sequences have little effect on levels of transcription, base changes in either of these elements substantially lower transcription rates (see, e.g., Myers et al., *Science*, 229, 242-247 (1985); McKnight et al., *Science*, 217, 316-324 (1982)). The position and orientation of these elements relative to one another, and to the start site, are important for the efficient transcription of some, but not all, coding sequences. For instance, some promoters function well in the absence of any TATA box. Similarly, the necessity of these and other sequences for promoters recognized by RNA polymerase I or III, or other RNA polymerases, can differ.

[0041] Accordingly, promoter regions can vary in length and sequence and can further encompass one or more DNA binding sites for sequence-specific DNA binding proteins

and/or an enhancer or silencer. Enhancers and/or silencers can similarly be present on a nucleic acid sequence outside of the promoter *per se*.

[0042] The present invention preferentially employs a viral promoter. Suitable viral promoters are known in the art and include, for instance, cytomegalovirus (CMV) promoters, such as the CMV immediate-early promoter, promoters derived from human immunodeficiency virus (HIV), such as the HIV long terminal repeat promoter, Rous sarcoma virus (RSV) promoters, such as the RSV long terminal repeat, mouse mammary tumor virus (MMTV) promoters, HSV promoters, such as the Lap2 promoter or the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci.*, 78, 144-145 (1981)), promoters derived from SV40 or Epstein Barr virus, an adeno-associated viral promoter, such as the p5 promoter, and the like. Preferably, the viral promoter is an adenoviral promoter, such as the Ad2 or Ad5 major late promoter and tripartite leader, a CMV promoter, or an RSV promoter.

[0043] Many of the above-described promoters are constitutive promoters. Instead of being a constitutive promoter, the promoter can be an inducible promoter, i.e., a promoter that is up- and/or down-regulated in response to appropriate signals. For instance, the regulatory sequences can comprise a hypoxia driven promoter, which is active when the ocular neovascularization or age-related macular degeneration is associated with hypoxia. Other examples of suitable inducible promoter systems include, but are not limited to, the IL-8 promoter, the metallothioneine inducible promoter system, the bacterial *lacZYA* expression system, the tetracycline expression system, and the T7 polymerase system. Further, promoters that are selectively activated at different developmental stages (e.g., globin genes are differentially transcribed from globin-associated promoters in embryos and adults) can be employed. The promoter sequence that regulates expression of the nucleic acid sequence can contain at least one heterologous regulatory sequence responsive to regulation by an exogenous agent. The regulatory sequences are preferably responsive to exogenous agents such as, but not limited to, drugs, hormones, or other gene products. For example, the regulatory sequences, e.g., promoter, preferably are responsive to glucocorticoid receptor-hormone complexes, which, in turn, enhance the level of transcription of a therapeutic peptide or a therapeutic fragment thereof.

[0044] Preferably, the regulatory sequences comprise a tissue-specific promoter, i.e., a promoter that is preferentially activated in a given tissue and results in expression of a gene product in the tissue where activated. A typically used tissue-specific promoter is a myocyte-specific promoter. A promoter exemplary of a myocyte-specific promoter is the myosin light-chain 1A promoter. A tissue specific promoter for use in the present inventive vector can be chosen by the ordinarily skilled artisan based upon the target tissue or cell-

type. Preferred tissue-specific promoters for use in the present inventive methods are specific to ocular tissue, such as a rhodopsin promoter. Examples of rhodopsin promoters include, but are not limited to, a GNAT cone- transducing alpha-subunit gene promoter or an interphotoreceptor retinoid binding protein promoter.

[0045] One of ordinary skill in the art will appreciate that each promoter drives transcription, and, therefore, protein expression, differently with respect to time and amount of protein produced. For example, the CMV promoter is characterized as having peak activity shortly after transduction, i.e., about 24 hours after transduction, then quickly tapering off. On the other hand, the RSV promoter's activity increases gradually, reaching peak activity several days after transduction, and maintains a high level of activity for several weeks. Indeed, sustained expression driven by an RSV promoter has been observed in all cell types studied, including, for instance, liver cells, lung cells, spleen cells, diaphragm cells, skeletal muscle cells, and cardiac muscle cells. Thus, a promoter can be selected for use in the methods of the present invention by matching its particular pattern of activity with the desired pattern and level of expression of at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Alternatively, a hybrid promoter can be constructed which combines the desirable aspects of multiple promoters. For example, a CMV-RSV hybrid promoter combining the CMV promoter's initial rush of activity with the RSV promoter's high maintenance level of activity would be especially preferred for use in many embodiments of the present inventive method. It is also possible to select a promoter with an expression profile that can be manipulated by an investigator.

[0046] Also preferably, the expression vector comprises a nucleic acid encoding a cis-acting factor, wherein the cis-acting factor modulates the expression of the nucleic acid sequence. Preferably, the cis-acting factor comprises matrix attachment region (MAR) sequences (e.g., immunoglobulin heavy chain (Jenunwin et al., *Nature*, 385(16), 269 (1997)), apolipoprotein B, or locus control region (LCR) sequences, among others. MAR sequences have been characterized as DNA sequences that associate with the nuclear matrix after a combination of nuclease digestion and extraction (Bode et al., *Science*, 255(5041), 195-197 (1992)). MAR sequences are often associated with enhancer-type regulatory regions and, when integrated into genomic DNA, MAR sequences augment transcriptional activity of adjacent nucleotide sequences. It has been postulated that MAR sequences play a role in controlling the topological state of chromatin structures, thereby facilitating the formation of transcriptionally-active complexes. Similarly, it is believed LCR sequences function to establish and/or maintain domains permissive for transcription. Many LCR sequences give tissue specific expression of associated nucleic acid sequences. Addition of

MAR or LCR sequences to the expression vector can further enhance expression of at least one inhibitor of angiogenesis and/or at least one neurotrophic factor.

[0047] With respect to promoters, nucleic acid sequences, selectable markers, and the like, located on an expression vector according to the present invention, such elements can be present as part of a cassette, either independently or coupled. In the context of the present invention, a "cassette" is a particular base sequence that possesses functions which facilitate subcloning and recovery of nucleic acid sequences (e.g., one or more restriction sites) or expression (e.g., polyadenylation or splice sites) of particular nucleic acid sequences.

[0048] Construction of an exogenous nucleic acid operably linked to regulatory sequences necessary for expression is well within the skill of the art (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989)). With respect to the expression of nucleic acid sequences according to the present invention, the ordinary skilled artisan is aware that different genetic signals and processing events control levels of nucleic acids and proteins/peptides in a cell, such as, for instance, transcription, mRNA translation, and post-transcriptional processing. Transcription of DNA into RNA requires a functional promoter, as described herein.

[0049] Protein expression is dependent on the level of RNA transcription that is regulated by DNA signals, and the levels of DNA template. Similarly, translation of mRNA requires, at the very least, an AUG initiation codon, which is usually located within 10 to 100 nucleotides of the 5' end of the message. Sequences flanking the AUG initiator codon have been shown to influence its recognition by eukaryotic ribosomes, with conformity to a perfect Kozak consensus sequence resulting in optimal translation (see, e.g., Kozak, *J. Molec. Biol.*, 196, 947-950 (1987)). Also, successful expression of an exogenous nucleic acid in a cell can require post-translational modification of a resultant protein. Thus, production of a protein can be affected by the efficiency with which DNA (or RNA) is transcribed into mRNA, the efficiency with which mRNA is translated into protein, and the ability of the cell to carry out post-translational modification. These are all factors of which the ordinary skilled artisan is aware and is capable of manipulating using standard means to achieve the desired end result.

[0050] Along these lines, to optimize protein production, preferably the nucleic acid sequence further comprises a polyadenylation site following the coding region of the nucleic acid sequence. Also, preferably all the proper transcription signals (and translation signals, where appropriate) will be correctly arranged such that the nucleic acid sequence will be properly expressed in the cells into which it is introduced. If desired, the nucleic acid sequence also can incorporate splice sites (i.e., splice acceptor and splice donor sites)

to facilitate mRNA production. Moreover, if the nucleic acid sequence encodes a protein or peptide, which is a processed or secreted protein or acts intracellularly, preferably the nucleic acid sequence further comprises the appropriate sequences for processing, secretion, intracellular localization, and the like.

[0051] In certain embodiments, it may be advantageous to modulate expression of the at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. An especially preferred method of modulating expression of a nucleic acid sequence comprises addition of site-specific recombination sites on the expression vector. Contacting an expression vector comprising site-specific recombination sites with a recombinase will either up- or down-regulate transcription of a coding sequence, or simultaneously up-regulate transcription one coding sequence and down-regulate transcription of another, through the recombination event. Use of site-specific recombination to modulate transcription of a nucleic acid sequence is described in, for example, U.S. Patents 5,801,030 and 6,063,627 and International Patent Application WO 97/09439.

[0052] Preferably, the expression vector of the present inventive method comprises a nucleic acid encoding an inhibitor of angiogenesis. More preferably, the nucleic acid sequence encodes multiple inhibitors of angiogenesis. By “inhibitor of angiogenesis” is meant any factor that prevents or ameliorates neovascularization. One of ordinary skill in the art will understand that complete prevention or amelioration of neovascularization is not required in order to realize a therapeutic effect. Therefore, the present inventive methods contemplate both partial and complete prevention and amelioration of angiogenesis. An inhibitor of angiogenesis includes, for instance, an anti-angiogenic factor, an anti-sense molecule specific for an angiogenic factor, a ribozyme, a receptor for an angiogenic factor, and an antibody that binds a receptor for an angiogenic factor.

[0053] The anti-angiogenic factors contemplated for use in the present invention include pigment epithelium-derived factor, angiostatin, vasculostatin, endostatin, platelet factor 4, heparinase, interferons (e.g., $\text{INF}\alpha$), and the like. One of ordinary skill in the art will appreciate that any anti-angiogenic factor can be modified or truncated and retain anti-angiogenic activity. As such, active fragments of anti-angiogenic factors (i.e., those fragments having biological activity sufficient to inhibit angiogenesis) are also suitable for use in the present inventive methods.

[0054] An anti-sense molecule specific for an angiogenic factor should generally be substantially identical to at least a portion, preferably at least about 20 continuous nucleotides, of the nucleic acid encoding the angiogenic factor to be inhibited, but need not be identical. The anti-sense nucleic acid molecule can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial

homology to the nucleic acid. The introduced anti-sense nucleic acid molecule also need not be full-length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the anti-sense molecule need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Antisense phosphorothiotac oligodeoxynucleotides (PS-ODNs) is exemplary of an anti-sense molecule specific for an angiogenic factor.

[0055] Ribozymes can be designed that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered and is, thus, capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff et al., *Nature*, 334, 585-591 (1988). Preferably, the ribozyme comprises at least about 20 continuous nucleotides complementary to the target sequence on each side of the active site of the ribozyme.

[0056] Receptors specific for angiogenic factors inhibit neovascularization by sequestering growth factors away from functional receptors capable of promoting a cellular response. For example, Flt and Flk receptors, as well as VEGF-receptor chimeric proteins, compete with VEGF receptors on vascular endothelial cells to inhibit endothelial cell growth (Aiello, *PNAS*, 92, 10457 (1995)). Also contemplated are growth factor-specific antibodies and fragments thereof (e.g., Fab, F(ab')₂, and Fv) that neutralize angiogenic factors or bind receptors for angiogenic factors.

[0057] The present invention also contemplates delivery of a nucleic acid sequence encoding at least one neurotrophic agent (or neurotrophic factor) to ocular cells or cells associated with age-related macular degeneration. Neurotrophic factors are thought to be responsible for the maturation of developing neurons and for maintaining adult neurons. Neurotrophic factors are divided into three subclasses: neuropoietic cytokines; neurotrophins; and the fibroblast growth factors. Ciliary neurotrophic factor (CNTF) is exemplary of neuropoietic cytokines. CNTF promotes the survival of ciliary ganglionic neurons and supports certain neurons that are NGF-responsive. Neurotrophins include, for example, brain-derived neurotrophic factor and nerve growth factor, perhaps the best characterized neurotrophic factor. Other neurotrophic factors suitable for being encoded by the nucleic acid sequence of the present inventive methods include, for example, transforming growth factors, glial cell-line derived neurotrophic factor, neurotrophin 3, neurotrophin 4/5, and interleukin 1- β . Neurotrophic factors associated with angiogenesis,

such as aFGF and bFGF, are less preferred. The neurotrophic factor of the present inventive method can also be a neuronotrophic factor, e.g., a factor that enhances neuronal survival. It has been postulated that neurotrophic factors can actually reverse degradation of neurons. Such factors, conceivably, are useful in treating the degeneration of neurons associated with vision loss. Neurotrophic factors function in both paracrine and autocrine fashions, making them ideal therapeutic agents. Preferably, the nucleic acid sequence of the present invention encodes both an inhibitor of angiogenesis and a neurotrophic factor. More preferably, the nucleic acid sequence encodes at least one factor comprising both anti-angiogenic and neurotrophic properties. Most preferably, the factor comprising both anti-angiogenic and neurotrophic properties is pigment epithelium-derived factor (PEDF).

[0058] PEDF, also named early population doubling factor-1 (EPC-1), is a secreted protein having homology to a family of serine protease inhibitors named serpins. PEDF is made predominantly by retinal pigment epithelial cells and is detectable in most tissues and cell types of the body. PEDF has been observed to induce differentiation in retinoblastoma cells and enhance survival of neuronal populations (Chader, *Cell Different.*, 20, 209-216 (1987)). Factors that enhance neuronal survival under adverse conditions, such as PEDF, are termed "neuronotrophic," as described herein. PEDF further has gliastatic activity, or has the ability to inhibit glial cell growth. As discussed above, PEDF also has anti-angiogenic activity. Anti-angiogenic derivatives of PEDF include SLED proteins, discussed in WO 99/04806. It has also been postulated that PEDF is involved with cell senescence (Pignolo et al., *J. Biol. Chem.*, 268(12), 8949-8957 (1998)). PEDF for use in the present inventive method can be derived from any source, and is further characterized in U.S. Patent 5,840,686 and International Patent Applications WO 93/24529 and WO 99/04806.

[0059] In addition to the methods of prophylactically or therapeutically treating an ocular-related disorder, the present invention further provides a viral vector comprising a nucleic acid sequence encoding PEDF or a therapeutic fragment thereof, wherein the nucleic acid sequence operably linked to regulatory sequences necessary for expression of PEDF or a therapeutic fragment thereof. The nucleic acid sequence can be obtained from any source, e.g., isolated from nature, synthetically generated, isolated from a genetically engineered organism, and the like. Appropriate viral vectors and regulatory sequences are discussed herein. In nature, PEDF is almost solely generated in human fetus retinal cells. The poor production of human PEDF from RPE cells and the scarcity of source tissue of PEDF complicates the use of this potentially valuable therapeutic factor. The viral vector of the present invention can be used to create sufficient amounts of recombinant PEDF or can be used in methods of research or treatment, e.g., the present inventive method.

[0060] The expression vector, e.g., the adenoviral or the adeno-associated viral vector, also can comprise a nucleic acid sequence encoding a therapeutic fragment of at least one inhibitor of angiogenesis or at least one neurotrophic factor. One of ordinary skill in the art will appreciate that any inhibitor of angiogenesis or neurotrophic factor, e.g., PEDF, can be modified or truncated and retain anti-angiogenic or neurotrophic activity. As such, therapeutic fragments (i.e., those fragments having biological activity sufficient to, for example, inhibit angiogenesis or promote neuron survival) also are suitable for incorporation into the expression vector. Also suitable for incorporation into the expression vector are nucleic acid sequences comprising substitutions, deletions, or additions, but which encode a functioning inhibitor of angiogenesis or neurotrophic factor or a therapeutic fragment of any of the foregoing. Likewise, a fusion protein comprising an anti-angiogenic factor or neurotrophic factor or a therapeutic fragment thereof and for example, a moiety that stabilizes peptide conformation, also can be present in the expression vector. A functioning inhibitor of angiogenesis or a therapeutic fragment thereof prevents or ameliorates neovascularization. A functioning neurotrophic factor or a therapeutic fragment thereof desirably promotes neuronal cell differentiation, inhibits glial cell proliferation, and/or promotes neuronal cell survival. One of ordinary skill in the art will understand that complete prevention or amelioration of neovascularization is not required in order to realize a therapeutic effect. Likewise, complete induction of neuron survival or differentiation is not required in order to realize a benefit. Therefore, both partial and complete prevention and amelioration of angiogenesis or promotion of neuron survival is appropriate. The ordinarily skilled artisan has the ability to determine whether a modified therapeutic factor or a fragment thereof has neurotrophic and anti-angiogenic therapeutic activity using, for example, neuronal cell differentiation and survival assays (see, for example, U.S. Patent 5,840,686), the mouse ear model of neovascularization, or the rat hindlimb ischemia model.

[0061] Similarly, one of ordinary skill in the art will appreciate that the inhibitor of angiogenesis and/or the neurotrophic factor can be a factor that acts upon a receptor for an anti-angiogenic factor or a receptor for a neurotrophic factor, thereby resulting in the desired biological effect. For instance, the expression vector can comprise a nucleic acid sequence encoding an antibody that binds and activates the PEDF receptor, which signals a series of intracellular events responsible for the biological activity of PEDF. For a discussion of PEDF receptors, see, for example, Alberdi et al., *J. Biol. Chem.*, 274(44), 31605 (1999).

[0062] The present invention also contemplates the use of nucleic acid sequences encoding chimeric or fusion peptides in the present inventive method. Through

recombinant DNA technology, scientists have been able to generate fusion proteins that contain the combined amino acid sequence of two or more proteins. The ordinarily skilled artisan can fuse the active domains of two or more factors to generate chimeric peptides with desired activity. The chimeric peptide can comprise the entire amino acid sequences of two or more peptides or, alternatively, can be constructed to comprise portions of two or more peptides (e.g., 10, 20, 50, 75, 100, 400, 500, or more amino acid residues). Desirably, the chimeric peptide comprises anti-angiogenic and neurotrophic activity, which can be determined using routine methods.

[0063] As discussed herein, the expression vector of the present inventive method comprises a nucleic acid sequence that encodes at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Therefore, the nucleic acid sequence can encode multiple, i.e., two, three, or more, inhibitors of angiogenesis. Likewise, the nucleic acid sequence can encode multiple, i.e., two, three, or more, neurotrophic factors. In a preferred embodiment, the nucleic acid sequence encodes PEDF and ciliary neurotrophic factor (CNTF). Also preferably, the nucleic acid sequence encodes at least one inhibitor of angiogenesis and at least one neurotrophic factor. Multiple inhibitors of angiogenesis and/or multiple neurotrophic factors can be operably linked to different promoters. As discussed herein, different promoters have dissimilar levels and patterns of activity. One of ordinary skill in the art will appreciate the freedom to dictate the expression of different coding sequences through the use of multiple promoters. Alternatively, the multiple coding sequences can be operably linked to the same promoter to form a polycistronic element. The polycistronic element is transcribed into a single mRNA molecule when transduced into the ocular cell. Translation of the mRNA molecule is initiated at each coding sequence, thereby producing the multiple, separate peptides simultaneously. The present invention also contemplates contacting an ocular cell or a cell associated with age-related macular degeneration with a cocktail of expression vectors, wherein each expression vector encodes a different inhibitor of angiogenesis and/or neurotrophic factor. The cocktail of expression vectors can further comprise different types of expression vectors, e.g., adenoviral vectors and adeno-associated viral vectors.

[0064] The methods of the present invention can be part of a treatment regimen involving other therapeutic modalities. It is appropriate, therefore, if the ocular-related disorder, namely ocular neovascularization or age-related macular degeneration, has been treated, is being treated, or will be treated with any of a number of ocular therapies, such as drug therapy, photodynamic therapy, photocoagulation laser therapy, panretinal therapy, thermotherapy, radiation therapy, or surgery. Preferably, the surgery is macular translocation, removal of subretinal blood, or removal of subretinal choroidal neovascular

membrane. The expression vector is preferably administered intraocularly for the prophylactic or therapeutic treatment of age-related macular degeneration or persistent or recurrent ocular neovascularization treated with surgery, laser photocoagulation, and photodynamic therapies.

[0065] The expression vector is preferably administered as soon as possible after it has been determined that an animal, such as a mammal, specifically a human, is at risk for ocular neovascularization or age-related macular degeneration (prophylactic treatment) or has begun to develop ocular neovascularization or age-related macular degeneration (therapeutic treatment). Treatment will depend, in part, upon the particular nucleic acid sequence used, the particular inhibitor of angiogenesis and/or neurotrophic factor expressed from the nucleic acid sequence, the route of administration, and the cause and extent, if any, of ocular neovascularization or age-related macular degeneration realized. For example, systemic administration or administration to both eyes is preferred in the prophylactic treatment of macular degeneration because, once one eye is affected, the other eye is at risk (up to 19% per year).

[0066] The expression vector of the present invention desirably is administered in a pharmaceutical composition, which comprises a pharmaceutically acceptable carrier and the expression vector(s). Any suitable pharmaceutically acceptable carrier can be used within the context of the present invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered and the particular method used to administer the composition.

[0067] Suitable formulations include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood or intracellular fluid of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Preferably, the pharmaceutically acceptable carrier is a buffered saline solution. More preferably, the expression vector for use in the present inventive methods is administered in a pharmaceutical composition formulated to protect the expression vector from damage prior to administration. For example, the pharmaceutical composition can be formulated to reduce loss of the expression vector on devices used to prepare, store, or administer the expression vector, such as glassware, syringes, or needles.

The pharmaceutical composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the expression vector. To this end, the pharmaceutical composition preferably comprises a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. Use of such a pharmaceutical composition will extend the shelf life of the vector, facilitate administration, and increase the efficiency of the present inventive methods. In this regard, a pharmaceutical composition also can be formulated to enhance transduction efficiency.

[0068] In addition, one of ordinary skill in the art will appreciate that the expression vector, e.g., viral vector, of the present invention can be present in a composition with other therapeutic or biologically-active agents. For example, therapeutic factors useful in the treatment of a particular indication can be present. For instance, if treating vision loss, hyaluronidase can be added to a composition to effect the break down of blood and blood proteins in the vitreous of the eye. Factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with *in vivo* administration of the viral vector and ocular distress. Immune system suppressors can be administered in combination with the present inventive method to reduce any immune response to the vector itself or associated with an ocular disorder. Anti-angiogenic factors, such as soluble growth factor receptors, growth factor antagonists, i.e., angiotensin, and the like also can be part of the composition, as well as additional neurotrophic factors. Similarly, vitamins and minerals, anti-oxidants, and micronutrients can be co-administered. Antibiotics, i.e., microbicides and fungicides, can be present to reduce the risk of infection associated with gene transfer procedures and other disorders.

[0069] One skilled in the art will appreciate that suitable methods, i.e., invasive and noninvasive methods, of administering an expression vector whereon the expression vector will contact an ocular cell are available. Although more than one route can be used to administer a particular expression vector, a particular route can provide a more immediate and more effective reaction than another route. Accordingly, the described routes of administration are merely exemplary and are in no way limiting.

[0070] The present inventive methods are not dependent on the mode of administering the expression vector to an animal, preferably a human, to achieve the desired effect. As such, any route of administration is appropriate so long as the expression vector contacts an appropriate ocular cell. The expression vector for use in the present inventive methods can be appropriately formulated and administered in the form of an injection, eye lotion, ointment, implant and the like. The expression vector can be applied, for example, systemically, topically, subconjunctivally, intraocularly, retrobulbarly, periocularly,

subretinally, or suprachoroidally. In certain cases, it may be appropriate to administer multiple applications and employ multiple routes, e.g., subretinal and intravitreal, to ensure sufficient exposure of ocular cells to the expression vector. Multiple applications of the expression vector may also be required to achieve the desired effect.

[0071] Depending on the particular case, it may be desirable to non-invasively administer the expression vector to a patient. For instance, if multiple surgeries have been performed, the patient displays low tolerance to anesthetic, or if other ocular-related disorders exist, topical administration of the expression vector may be most appropriate. Topical formulations are well known to those of skill in the art. Such formulations are suitable in the context of the present invention for application to the skin. The use of patches, corneal shields (see, e.g., U.S. Patent 5,185,152), and ophthalmic solutions (see, e.g., U.S. Patent 5,710,182) and ointments, e.g., eye drops, is also within the skill in the art. The expression vector can also be administered non-invasively using a needleless injection device, such as the Biojector 2000 Needle-Free Injection Management System® available from Bioject, Inc.

[0072] The expression vector is preferably present in or on a device that allows controlled or sustained release of the expression vector, such as an ocular sponge, meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patents 5,443,505, 4,853,224 and 4,997,652), devices (see, e.g., U.S. Patents 5,554,187, 4,863,457, 5,098,443 and 5,725,493), such as an implantable device, e.g., a mechanical reservoir, an intraocular device or an extraocular device with an intraocular conduit, or an implant or a device comprised of a polymeric composition are particularly useful for ocular administration of the expression vector. The expression vector of the present inventive methods can also be administered in the form of sustained-release formulations (see, e.g., U.S. Patent 5,378,475) comprising, for example, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), or a polylactic-glycolic acid.

[0073] Alternatively, the expression vector can be administered using invasive procedures, such as, for instance, intravitreal injection or subretinal injection optionally preceded by a vitrectomy. Subretinal injections can be administered to different compartments of the eye, i.e., the anterior chamber. While intraocular injection is preferred, injectable compositions can also be administered intramuscularly, intravenously, and intraperitoneally. Pharmaceutically acceptable carriers for injectable compositions are well-known to those of ordinary skill in the art (see *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Co., Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)).

Although less preferred, the expression vector can also be administered *in vivo* by particle bombardment, i.e., a gene gun.

[0074] Preferably, the expression vector is administered via an ophthalmologic instrument for delivery to a specific region of an eye. Use of a specialized ophthalmologic instrument ensures precise administration of the expression vector while minimizing damage to adjacent ocular tissue. Delivery of the expression vector to a specific region of the eye also limits exposure of unaffected cells to the inhibitor of angiogenesis and/or neurotrophic factor, thereby reducing the risk of side effects. A preferred ophthalmologic instrument is a combination of forceps and subretinal needle or sharp bent cannula.

[0075] While not particularly preferred, the expression vector can be administered parenterally. Preferably, any expression vector parenterally administered to a patient for the prophylactic or therapeutic treatment of an ocular-related disorder, i.e., ocular neovascularization or age-related macular degeneration, is specifically targeted to ocular cells. As discussed herein, an expression vector can be modified to alter the binding specificity or recognition of an expression vector for a receptor on a potential host cell. With respect to adenovirus, such manipulations can include deletion of regions of the fiber, penton, or hexon, insertions of various native or non-native ligands into portions of the coat protein, and the like. One of ordinary skill in the art will appreciate that parenteral administration can require large doses or multiple administrations to effectively deliver the expression vector to the appropriate host cells.

[0076] One of ordinary skill in the art will also appreciate that dosage and routes of administration can be selected to minimize loss of expression vector due to a host's immune system. For example, for contacting ocular cells *in vivo*, it can be advantageous to administer to a host a null expression vector (i.e., an expression vector not comprising the nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor) prior to performing the present inventive method. Prior administration of null expression vectors can serve to create an immunity in the host to the expression vector, thereby decreasing the amount of vector cleared by the immune system.

[0077] The dose of expression vector administered to an animal, particularly a human, in accordance with the present invention should be sufficient to effect the desired response in the animal over a reasonable time frame. One skilled in the art will recognize that dosage will depend upon a variety of factors, including the age, species, the pathology in question, and condition or disease state. Dosage also depends on the inhibitor of angiogenesis and/or neurotrophic factor to be expressed, as well as the amount of ocular tissue about to be affected or actually affected by the ocular-related disease. The size of the dose also will be determined by the route, timing, and frequency of administration as well

as the existence, nature, and extent of any adverse side effects that might accompany the administration of a particular expression vector and the desired physiological effect. It will be appreciated by one of ordinary skill in the art that various conditions or disease states, in particular, chronic conditions or disease states, may require prolonged treatment involving multiple administrations.

[0078] Suitable doses and dosage regimens can be determined by conventional range-finding techniques known to those of ordinary skill in the art. Preferably, the about 10^6 viral particles to about 10^{12} viral particles are delivered to the patient. In other words, a pharmaceutical composition can be administered that comprises an expression vector concentration of from about 10^6 particles/ml to about 10^{12} particles/ml (including all integers within the range of about 10^6 particles/ml to about 10^{12} particles/ml), preferably from about 10^{10} particles/ml to about 10^{12} particles/ml, and will typically involve the intraocular administration of from about 0.1 μ l to about 100 μ l of such a pharmaceutical composition per eye. When the expression vector is a plasmid, preferably about 0.5 ng to about 1000 μ g of DNA is administered. More preferably, about 0.1 μ g to about 500 μ g is administered, even more preferably about 1 μ g to about 100 μ g of DNA is administered. Most preferably, about 50 μ g of DNA is administered per eye. Of course, other routes of administration may require smaller or larger doses to achieve a therapeutic effect. Any necessary variations in dosages and routes of administration can be determined by the ordinarily skilled artisan using routine techniques known in the art.

[0079] In some embodiments, it is advantageous to administer two or more (i.e., multiple) doses of the expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic agent. The present inventive method provides for multiple applications of the inhibitor of angiogenesis and/or neurotrophic agent to prophylactically or therapeutically treat ocular neovascularization or other ocular-related disorders. For example, at least two applications of an expression vector comprising an exogenous nucleic acid, e.g., a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic agent, can be administered to the same eye. Preferably, the multiple doses are administered while retaining gene expression above background levels. Also preferably, the ocular cell is contacted with two applications or more of the expression vector within about 30 days or more. More preferably, two or more applications are administered to ocular cells of the same eye within about 90 days or more. However, three, four, five, six, or more doses can be administered in any time frame (e.g., 2, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 85 or more days between doses) so long as gene expression occurs and ocular neovascularization is inhibited or ameliorated.

[0080] It also will be appreciated by one skilled in the art that an expression vector comprising a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor can be introduced *ex vivo* into cells, preferably ocular cells, previously removed from a given animal, in particular a human. Such transduced autologous or homologous host cells, reintroduced into the animal or human, will express directly at least one inhibitor of angiogenesis and/or at least one neurotrophic factor *in vivo*. One *ex vivo* therapeutic option involves the encapsidation of infected ocular cells into a biocompatible capsule, which can be implanted in the eye or any other part of the body. One of ordinary skill in the art will understand that such cells need not be isolated from the patient, but can instead be isolated from another individual and implanted into the patient.

[0081] It will be appreciated that the expression vector, preferably the adenoviral vector, can comprise multiple nucleic acid sequences encoding the at least one inhibitor of angiogenesis and/or the at least one neurotrophic factor. For example, the expression vector can comprise multiple copies of the PEDF coding sequence, each copy operably linked to a different promoter or to identical promoters.

[0082] In addition to the above, the nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic factor can further comprise one or more other transgenes. By "transgene" is meant any nucleic acid that can be expressed in a cell. Desirably, the expression of the transgene is beneficial, e.g., prophylactically or therapeutically beneficial, to the ocular cell or eye. If the transgene confers a prophylactic or therapeutic benefit to the cell, the transgene can exert its effect at the level of RNA or protein. For example, the transgene can encode a peptide other than an inhibitor of angiogenesis or neurotrophic factor that can be employed in the treatment or study of a disorder, e.g., an ocular-related disorder. Alternatively, the transgene can encode an antisense molecule, a ribozyme, a protein that affects splicing or 3' processing (e.g., polyadenylation), or a protein that affects the level of expression of another gene within the cell (i.e., where gene expression is broadly considered to include all steps from initiation of transcription through production of a process protein), such as by mediating an altered rate of mRNA accumulation or transport or an alteration in post-transcriptional regulation. The transgene can encode a chimeric peptide for combination treatment of an ocular-related disorder. The transgene can encode a factor that acts upon a different target molecule than the inhibitor of angiogenesis or the neurotrophic agent. Indeed, the transgene product can act upon a different signal transduction pathway, or can act at different points of the same signal transduction pathway of the inhibitor of angiogenesis or the neurotrophic factor. Preferably, the therapeutic substance is a neurotrophic factor, such as CNTF. CNTF belongs to the neuropoietic cytokines subclass of neurotrophic factors. CNTF promotes the

survival of ciliary ganglionic neurons and supports certain neurons that are nerve growth factor (NGF)-responsive.

[0083] Alternatively, one or more additional nucleic acid sequences (e.g., transgenes) that encode a factor associated with cell differentiation can be included in the expression vector (e.g., viral vector). Preferably, the transgene encodes an atonal-associated peptide such as Math1 or Hath1 or a biologically active fragment of either of the foregoing. Math1 is a member of the mouse basic helix-loop-helix family of transcription factors and is homologous to the *Drosophila* gene *atonal*. Hath1 is the human counterpart of Math1. Math1 has been shown to be essential for hair development and can stimulate hair regeneration in the ear. Combining neurotrophic activity and the hair cell differentiation properties of an atonal-associated peptide provides a powerful tool for the treatment and research of, for example, sensory disorders. Math1 is further characterized in, for example, Bermingham et al., *Science*, 284, 1837-1841 (1999) and Zheng and Gao, *Nature Neuroscience*, 3(2), 580-586 (2000).

[0084] The expression vector can comprise a nucleic acid sequence encoding a vessel maturation factor in addition to at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. Many ocular disorders involve leakage of blood products through vessels, which can cloud vision and induce an immune response within the layers of the eye. Vessel maturation factors reduce the amount of vascular leakage and, therefore, are useful in treating, for example, exudative ocular disorders. Vessel maturation factors include, but are not limited to, angiopoietins (Ang, e.g., Ang-1 and Ang-2), tumor necrosis factor-alpha (TNF- α), midkine (MK), COUP-TFII, and heparin-binding neurotrophic factor (HBNF, also known as heparin binding growth factor). A nucleotide sequence encoding an immunosuppressor also can be incorporated into the expression vector to reduce any inappropriate immune response within the eye as a result of an ocular-related disorder or the administration of the expression vector.

[0085] The expression vector can comprise one or more additional nucleic acid sequences encoding an additional anti-angiogenic substance. As set forth above, an anti-angiogenic substance is any biological factor that prevents or ameliorates neovascularization. One of ordinary skill in the art will understand that the anti-angiogenic substance can effect partial or complete prevention and amelioration of angiogenesis to achieve a therapeutic effect. An anti-angiogenic substance includes, for instance, an anti-angiogenic factor, an anti-sense molecule specific for an angiogenic factor, a ribozyme, a receptor for an angiogenic factor, and an antibody that binds a receptor for an angiogenic factor.

[0086] The transgene can encode a marker protein, such as green fluorescent protein or luciferase. Such marker proteins are useful in vector construction and determining vector migration. Marker proteins also can be used to determine points of injection or treated ocular tissues in order to efficiently space injections of the expression vector to provide a widespread area of treatment, if desired. Alternatively, the transgene can encode a selection factor, which also is useful in vector construction protocols. If desired, the transgene can be part of an expression cassette.

[0087] It should be appreciated that any of the nucleic acid sequences described herein can be altered from their native form to increase their therapeutic effect. For example, a cytoplasmic form of a therapeutic nucleic acid can be converted to a secreted form by incorporating a signal peptide into the encoded gene product. The at least one inhibitor of angiogenesis and/or at least one neurotrophic factor can be designed to be taken up by neighboring cells by fusion of the peptide with VP22. This allows an ocular cell comprising the therapeutic nucleic acid to have a therapeutic effect on a number of ocular cells within the mammal.

[0088] The present inventive methods also can involve the co-administration of other pharmaceutically active compounds. By "co-administration" is meant administration before, concurrently with, e.g., in combination with the expression vector in the same formulation or in separate formulations, or after administration of the expression vector as described above. For example, factors that control inflammation, such as ibuprofen or steroids, can be co-administered to reduce swelling and inflammation associated with intraocular administration of the expression vector. Immunosuppressive agents can be co-administered to reduce inappropriate immune responses related to an ocular disorder or the practice of the present inventive method. Anti-angiogenic factors, such as soluble growth factor receptors, growth factor antagonists, i.e., angiotensin, and the like can also be co-administered, as well as neurotrophic factors. Similarly, vitamins and minerals, anti-oxidants, and micronutrients can be co-administered. Antibiotics, i.e., microbicides and fungicides, can be co-administered to reduce the risk of infection associated with ocular procedures and some ocular-related disorders.

[0089] Although the expression vectors of the instant invention are particularly useful in the study or treatment of ocular disorders, including ocular disorders comprising a neovascular component and age-related macular degeneration, it will be appreciated that the expression vectors can be used to research and/or treat prophylactically or therapeutically a wide variety of animal diseases. For example, the viral vector comprising a nucleic acid sequence encoding PEDF or a therapeutic fragment thereof can be used in the study or treatment of the nervous system, genitourinary ailments, cancer, infectious disease, and

cardiovascular abnormalities, as well as miscellaneous other health nuisances. The viral vector can be used to study or treat, for example, sleep disorders, ALS (Lou Gehrig's Disease), Alzheimer's Disease, epilepsy, multiple sclerosis, Parkinson's Disease, peripheral neuropathies, Schizophrenia, depression, anxiety, spinal cord injury, traumatic brain injury, or acute, chronic, or inflammatory pain. The expression vectors of the present invention can be used to treat genitourinary ailments, which include, for example, benign prostatic hyperplasia (BPH), impotence, neurogenic bladder, urinary incontinence, kidney failure, and end stage renal disease. The expression vectors are useful in treating cancer such as, for example, cancer of the bladder, brain, breast, colorectal, esophageal, head & neck, liver/hepatoma, lung, melanoma, ovarian, pancreatic, prostate, stomach, testicular, uterine/endometrial, leukemias, and lymphomas. Exemplary infectious diseases for treatment with the expression vectors include, but are not limited to, chlamydia, herpes, malaria, human papilloma virus (HPV), AIDS/HIV, pneumococcal pneumonia, influenza, meningitis, hepatitis, and tuberculosis. Cardiovascular diseases such as, for example, neovascular diseases, ischemia, congestive heart failure, coronary artery disease, arrhythmia, atherosclerosis, increased LDL/HDL ratios, restenosis after angioplasty or in-stent restenosis, stroke, sickle cell anemia, and hemophilia, can be treated or studied, as well as the alleviation of, for example, obesity, organ transplantation/transplant rejection, osteoporosis, alopecia, hair loss, arthritis, allergies (such as to ragweed, pollen, and animal dander), cystic fibrosis, diabetes, and hearing loss. One of ordinary skill in the art will appreciate that animal models exist for many of the disease states identified above.

EXAMPLES

[0090] The following examples further illustrate the present invention but, of course, should not be construed as in any way limiting its scope.

Example 1

[0091] This example illustrates a preferred method of obtaining expression of a factor comprising both anti-angiogenic and neurotrophic activity from an adenoviral vector in *in vivo* retina.

[0092] An adenoviral vector deficient in one or more essential gene functions of the E1, E3, and E4 regions of the adenoviral genome and comprising a PEDF gene (Ad.PEDF) is preferably constructed as set forth in WO 99/15686 (McVey et al.). However, the method of the present invention is not dependent on the method of vector construction employed and previously described methods of vector construction are also suitable.

Several *in vivo* models of ocular neovascularization are available. Neovascularization of the retina is obtained in, for example, neonatal animals, i.e., neonatal mice, by exposing the mice to hypoxic conditions shortly after birth. Several days later, the neonatal mice are exposed to standard atmospheric conditions, resulting in ischemia-induced neovascularization of the retina.

[0093] Ad.PEDF is administered to the right eye of at least 12 day old mice anesthetized with, for example, ketamine or a combination of ketamine and xylazine via intravitreal injection. Injections are performed by forming an entrance site in the posterior portion of the eye and administering approximately 0.1-5.0 μ l of composition comprising Ad.PEDF. In most instances, an injection of the expression vector will be administered to only one eye, while the remaining eye serves as a control. The mice are sacrificed at various time points after administration to determine the extent and duration of PEDF expression in the retina. The right and left eyes of each animal are enucleated and either fixed for histological analysis or prepared for PEDF expression analysis. Detection of PEDF DNA, PEDF RNA, or PEDF protein can be accomplished using methods well known in the art, such as PCR and blotting techniques (see, for example, Sambrook et al., *supra*).

[0094] To determine the effect of PEDF on neovascularization *in vivo* in, for example, a human, indirect ophthalmoscopy of the retina is ideal. Stereophotographs are useful in detecting extensive neovascularization, but not appropriate for detecting subtle lesions.

Example 2

[0095] This example demonstrates a preferred method of obtaining expression of a factor comprising both anti-angiogenic and neurotrophic activity from an adenoviral vector in *in vivo* choroid. The following example further provides methods for determining the effect of PEDF on neovascularization.

[0096] An adenoviral vector deficient in one or more essential gene functions of the E1, E3, and E4 regions of the adenoviral genome and comprising a PEDF gene (Ad.PEDF) is constructed as set forth in WO 99/15686 (McVey et al.).

[0097] An *in vivo* model of choroidal neovascularization can be obtained by detaching the retina of an eye of, for example, a mouse or rabbit, and debriding the pigmented epithelia. Choriocapillary regeneration is monitored in both treated and untreated eyes. Ad.PEDF is administered prior to perturbing the retinal pigment epithelial (RPE) to determine the effect of the present inventive method in preventing choroidal neovascularization. Of course, Ad.PEDF is administered after perturbing the retina and RPE for determining the therapeutic effect of the procedure on neovascularization.

[0098] Choroidal neovascularization can be monitored *in vivo* using fundus photography, fluorescein angiography and/or indocyanine-green angiography, as commonly used in the art. Using these methods, one of ordinary skill in the art is able to detect growth of new blood vessels and vascular leakage often associated with neovascularization. For research purposes, neovascularization can also be determined by enucleating the eyes and preparing vascular casts or examining ocular tissue via scanning electron microscopy.

Example 3

[0099] This example demonstrates the utility of adenoviral vectors to deliver multiple doses of an exogenous nucleic acid to the eye.

[0100] Adenoviral vectors comprising the luciferase gene (Ad.L) or control adenoviral vectors comprising no transgene (Ad.null) were injected into the intravitreal space of C57BL6 mouse eyes (Day 0). One day following injection of the adenoviral vectors (Day 1), eyes infected with Ad.L were enucleated and frozen (1st administration). The eyes infected with Ad.null were divided into three groups. In Group I, Ad.L vectors were injected into the intravitreal space of the eye at Day 14 (fourteen days following the initial dose of Ad.null). Group I eyes were enucleated and frozen the day following the second administration of adenoviral vectors (Day 15, 2nd administration). Group II eyes were injected intravitreally with Ad.null at Day 14, and injected intravitreally with Ad.L vectors four weeks following the initial injection with Ad.null (Day 28, 3rd administration). The eyes were then enucleated and frozen the day after the third administration of adenoviral vector. Group III eyes were injected intravitreally with Ad.null at Day 14 and Day 28, and injected with Ad.L vectors six weeks following the initial injection with Ad.null (Day 42, 4th administration). The eyes were then enucleated and frozen the day after the fourth administration of adenoviral vector. Luciferase assays were performed on the eye samples to determine the efficiency of infection and gene expression associated with multiple dosing of the vectors.

[0101] Luciferase expression in ocular cells after the 1st and 2nd administration of adenoviral vector was substantially equivalent. In other words, no loss of gene expression was detected following two administrations of the gene transfer vector. Gene expression from the 3rd administration of adenoviral vector was between 10- and 100-fold reduced compared to gene expression from the 1st administration and the 2nd administration, but was still above background levels (e.g., as detected in cells transduced with Ad.null). Gene expression from the 4th administration of adenoviral vector was reduced approximately 3- to 10-fold compared to the gene expression observed following the 3rd

administration. However, the level of gene expression following the 4th administration was above background levels.

[0101] This example demonstrates the feasibility of performing multiple applications of adenoviral vectors to the eye in order to obtain expression of an exogenous nucleic acid in ocular cells.

Example 4

[0102] This example demonstrates the ability of an expression vector comprising a nucleic acid sequence encoding a factor comprising both anti-angiogenic and neurotrophic properties to inhibit choroidal neovascularization (CNV).

[0103] Replication-deficient (E1-/E3-deficient) adenoviral vectors (AdPEDF.10) comprising the coding sequence for PEDF operably linked to the CMV immediate early promoter were constructed using standard techniques. A null version of the vector (AdNull.10), which did not comprise the PEDF coding sequence, was also constructed.

[0104] Adult C57BL/6 mice were injected intravitreally with AdNull.10 or AdPEDF.10 using a Harvard pump microinjection apparatus and pulled glass micropipettes. Each eye was injected intravitreally with 1 μ l of vehicle containing 10^9 particles of virus. Alternatively, each eye was injected subretinally with 10^8 particles of virus suspended in 1 μ l of vehicle. Five days post-injection, mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight). Topiramide (1%) was utilized to dilate the pupils prior to rupture of Bruch's membrane by diode laser photocoagulation. Rupture of Bruch's membrane is known to induce neovascularization of the choroid.

[0105] Fourteen days following laser-induced rupture of Bruch's membrane, choroidal flat mounts (described in Edelman et al., *Invest. Ophthalmol. Vis. Sci.*, 41, S834 (2000)) were prepared to observe the degree of neovascularization of the choroidal membrane. Briefly, eyes were removed from the subjects and fixed in phosphate-buffered formalin. The cornea, lens, and retina were removed from the eyecup, and the eyecup was flat-mounted. Flat mounts were then examined by fluorescence microscopy and images were digitized using a 3 color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) for computer image analysis.

[0106] Large areas of neovascularization were observed in uninjected eyes and eyes receiving AdNull.10. Eyes injected with AdPEDF.10 subretinally or intravitreally showed smaller regions of neovascularization compared to the controls using computerized image analysis.

[0107] The above results illustrate the ability of the present inventive method to inhibit ocular neovascularization, namely choroidal neovascularization (CNV), in a clinically animal relevant model.

Example 5

[0108] This example demonstrates the ability of an expression vector comprising a nucleic acid sequence encoding a factor comprising both anti-angiogenic and neurotrophic properties to inhibit ischemia-induced retinal neovascularization.

[0109] Replication-deficient adenoviral vectors comprising the coding sequence for PEDF operably linked to the CMV immediate early promoter were constructed using standard techniques. E1-/E3-/E4-deficient vectors encoding PEDF (AdPEDF.11) and a null version of the vector (AdNull.11), which did not comprise the PEDF coding sequence, were constructed.

[0110] Ischemic retinopathy was produced in adult C57BL/6 mice as previously described (see, for example, Smith et al., *Invest. Ophthalmol. Vis. Sci.*, 35, 101 (1994)). Briefly, seven day old mice (P7) were exposed to an atmosphere of 75 +/- 3% oxygen for five days. At P10, mice were injected intravitreally with 10^9 particles of AdPEDF.11 or AdNull.11, returned to oxygen for two days, then returned to room atmosphere. At P17, the mice were sacrificed and eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN).

[0111] To detect neovascularization, the eyes were sectioned and histochemically stained with biotinylated griffonia simplicifolia lectin B4 (GSA, Vector Laboratories, Burlingame, CA). Slides were then incubated in methanol/H₂O₂ for 10 minutes at 4 °C, washed with 0.05 M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 minutes in 10% normal porcine serum. The slides were then incubated for two hours with biotinylated GSA, rinsed with TBS, and incubated with avidin-coupled alkaline phosphatase (Vector Laboratories) for 45 minutes. After a 10 minute wash with TBS, the slides were incubated with Histomark Red. GSA-stained, 10 µm serial sections were examined using an Axioskop microscope. Images were digitized using a 3 color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) for computer image analysis.

[0112] Extensive retinal neovascularization was detected in eyes not injected with any virus. Eyes injected with AdNull.11 showed less neovascularization than uninjected eyes, but significantly more neovascularization of the retina than eyes injected with AdPEDF.11. Eyes injected with AdPEDF.11 comprised the least amount of neovascularization.

[0113] This example clearly demonstrates the ability of the present inventive method to inhibit an ocular-related disorder, namely ischemia-induced retinal neovascularization, in a clinically relevant animal model.

[0114] All references cited herein are hereby incorporated by reference to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and was set forth in its entirety herein.

[0115] While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of prophylactically or therapeutically treating an animal for an ocular-related disorder, wherein the method comprises contacting an ocular cell with
 - (a) an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent, or
 - (b) different expression vectors, each comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a nucleic acid sequence encoding a neurotrophic agent,such that the nucleic acid sequence encoding the inhibitor of angiogenesis and/or the nucleic acid sequence encoding the neurotrophic agent are expressed, thereby resulting in the production of the inhibitor of angiogenesis and/or the neurotrophic agent to prophylactically or therapeutically treat the animal for an ocular-related disorder.
2. The method of claim 1, wherein the method comprises contacting the ocular cell with an expression vector comprising the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent.
3. The method of claim 2, wherein the nucleic acid sequence encoding the inhibitor of angiogenesis and the nucleic acid sequence encoding the neurotrophic agent are the same nucleic acid sequence.
4. The method of claim 1, wherein the method comprises contacting the ocular cell with at least two different types of expression vectors, wherein each expression vector comprises a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a nucleic acid sequence encoding a neurotrophic factor.
5. The method of any of claims 1-4, wherein the ocular-related disorder is ocular neovascularization.
6. The method of claim 5, wherein the ocular neovascularization is neovascularization of the choroid.
7. The method of claim 5, wherein the ocular neovascularization is neovascularization of the retina.

8. The method of claim 7, wherein the neovascularization of the retina is associated with diabetic retinopathy.

9. The method of any of claims 1-4, wherein the ocular-related disorder is age-related macular degeneration.

10. The method of any of claims 1-9, wherein at least one expression vector is an adeno-associated vector.

11. The method of any of claims 1-9, wherein at least one expression vector is an adenoviral vector.

12. The method of any of claims 1-9, wherein at least one expression vector is an adenoviral vector and at least one expression vector is an adeno-associated viral vector.

13. The method of claim 11 or claim 12, wherein the adenoviral vector is replication deficient.

14. The method of any of claims 1-13, wherein the expression vector(s) is (are) administered to cells of neural origin, ciliary epithelial cells, retinal pigment epithelial cells, glial cells, fibroblasts, endothelial cells, or cells of the trabecular meshwork.

15. The method of any of claims 1-13, wherein the expression vector(s) is (are) administered to iris epithelial cells, corneal cells, ciliary epithelial cells, Mueller cells, or astrocytes.

16. The method of any one of claims 1-15, wherein the expression vector(s) is (are) administered to a patient greater than 55 years of age.

17. The method of any one of claims 1-16, wherein the expression vector(s) is (are) administered to an area of vascular leakage.

18. The method of any of claims 1-17, wherein the expression vector(s) is present in or on a device that allows controlled release of the expression vector(s).

19. The method of any of claims 1-18, wherein the expression vector(s) is (are) administered topically, subconjunctivally, retrobulbarly, periorcularly, subretinally, suprachoroidally, or intraocularly.

20. The method of any of claims 4-19, wherein at least one expression vector comprises the nucleic acid sequence encoding an inhibitor of angiogenesis.

21. The method of any of claims 1-20, wherein the inhibitor of angiogenesis is selected from the group consisting of an anti-angiogenic factor, an anti-sense molecule specific for an angiogenic factor, a ribozyme, and a receptor for an angiogenic factor.

22. The method of any of claims 1-21, wherein the nucleic acid sequence encoding the inhibitor of angiogenesis encodes multiple inhibitors of angiogenesis.

23. The method of any of claims 4-22, wherein at least one expression vector comprises the nucleic acid sequence encoding a neurotrophic factor.

24. The method of any of claims 4-23, wherein at least one expression vector comprises the nucleic acid sequence encoding an inhibitor of angiogenesis and the nucleic acid sequence encoding a neurotrophic agent.

25. The method of any of claims 3-24, wherein the inhibitor of angiogenesis and the neurotrophic agent are a single factor.

26. The method of any of claims 1-25, wherein the neurotrophic agent is pigment epithelial-derived factor.

27. The method of any of claims 1-26, wherein the method comprises administering the expression vector(s) in two or more applications to the same eye of the animal.

28. A viral vector comprising a nucleic acid sequence encoding pigment epithelium-derived factor or a therapeutic fragment thereof, wherein the nucleic acid sequence is operably linked to regulatory sequences necessary for expression of pigment epithelium-derived factor or a therapeutic fragment thereof.

29. The viral vector of claim 28, wherein the viral vector is an adenoviral vector.
30. The viral vector of claim 29, wherein the adenoviral vector is replication deficient.
31. The viral vector of claim 30, wherein the adenoviral vector is lacking all or part of the E1 region.
32. The viral vector of claim 31, wherein the adenoviral vector is lacking all or part of the E1a region and/or is lacking all or part of the E1b region.
33. The viral vector of any of claims 30-32, wherein the adenoviral vector is lacking all or part of the E4 region.
34. The viral vector of any of claims 31-33, wherein the adenoviral vector is lacking all or part of the E3 region.
35. The viral vector of claim 28, wherein the viral vector is an adeno-associated vector.
36. The viral vector of any of claims 28-35 further comprising one or more additional nucleic acid sequences encoding therapeutic substances other than pigment epithelium-derived factor or a therapeutic fragment thereof.
37. The viral vector of claim 36, wherein one or more additional nucleic acid sequences encodes ciliary neurotrophic factor.
38. The viral vector of claim 36, wherein one or more additional nucleic acid sequences encodes an atonal-associated peptide.
39. The viral vector of claim 36, wherein one or more additional nucleic acid sequences encodes an anti-angiogenic substance.